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ANTI-*HELICOBACTER* VACCINE COMPOSITION FOR USE BY THE SUBDIAPHRAGMATIC SYSTEMIC ROUTE, AND COMBINED MUCOSAL/PARENTERAL IMMUNIZATION METHOD

The subject of the present invention is the specific use of a vaccine preparation intended to induce, in a mammal, a protective immune response against a pathogenic organism infecting the mucous membranes, in particular against *Helicobacter* bacteria.

Helicobacter is a bacterial genus characterized by Gram-negative helical bacteria. Several species colonize the gastrointestinal tract of mammals. There may be mentioned in particular *H. pylori, H. heilmanii, H. felis*, and *H. mustelae*. Although *H. pylori* is the species most commonly associated with human infections, in some rare cases, it has been possible to isolate in man *H. heilmanii* and *H. felis*. A bacterium of the *Helicobacter* type, *Gastrospirillum hominis*, has also been described in man.

Helicobacter infects more than 50% of the adult population in developed countries and nearly 100% of that of developing countries, thereby making it one of the predominant infectious agents worldwide.

H. pylori is so far exclusively found at the surface of the mucous membrane of the stomach in man and more particularly around the crater lesions of gastric and duodenal ulcers. This bacterium is currently recognized as the aetiological agent of antral gastritis and appears as one of the cofactors required for the development of ulcers. Moreover, it seems that the development of gastric carcinomas may be associated with the presence of *H. pylori*.

It therefore appears to be highly desirable to develop a vaccine intended to prevent or treat *Helicobacter* infections.

To date, several *Helicobacter* proteins have already been proposed as vaccinal antigens and the method of vaccination that is commonly recommended consists of

delivering the antigen at the level of the gastric mucous membrane, that is to say at the very site where the immune response is desired. To do this, oral administration was therefore selected.

Still with the same aim, induction of an immune response at the level of the stomach, it has been more recently proposed to deliver the antigen at a mucosal site other than the gastric mucous membrane, such as the nasal or rectal mucous membrane, for example (WO 96/31235). Lymphocytes stimulated by the antigen in a so-called inducer mucosal territory can migrate and circulate selectively so as to go and induce an immune response in other so-called effector mucosal territories.

A variant of these methods involves carrying out a first immunization by the systemic route before administering the antigen by the nasal route.

For administration by the mucosal route, the antigen, most often a bacterial lysate or a purified protein, is combined with an appropriate adjuvant such as cholera toxin (CT) or the heat-labile toxin (LT) from *E. coli.*

When administration by the mucosal route is used, the humoral response that is observed is predominantly of the IgA type. This indeed indicates that there has been a local immune response.

Some authors thought very early on that there was a good correlation between a strong response of the IgA type and a protective effect (Czinn *et al.*, Vaccine (1993) 11: 637). Others gave a more reserved opinion (Bogstedt *et al.*, Clin. Exp. Immunol. (1996) 105: 202). Although there is up until now no real certainty on this subject, the induction of antibodies that are in particular of the IgA type appears nonetheless desirable for most authors.

In general, the appearance of IgAs is indicative of the coming into play of a response on the part of the type 2 T helper lymphocytes (Th2 response).

Indeed, the stimulation of the T helper lymphocytes by a particular antigen makes it possible to obtain various subpopulations of T helper cells, characterized by different cytokine synthesis profiles.

The Th1 cells in particular produce selectively interleukin-2 (IL-2) and interferon-γ (IFN-γ), whereas the Th2 cells secrete preferably IL-4, IL-5, and IL-10. Because of their differentiated production of cytokines, these two types of T helper cells have distinct roles: the Th1 cells promote cell-mediated immunity, *i.a.*, an inflammatory-type response, whereas the Th2 cells stimulate humoral response of the IgA, IgE, and certain IgG subclass types. It is also known that the cytokines produced by mouse Th1 cells can stimulate antibody response and in particular that IFN-γ induces an IgG2a response. Thus, from the various studies in the prior art, the view emerges according to which the induction of a Th2 response characterized by the appearance of IgA is essential, if not enough, to obtain a protective effect.

Surprisingly, it has now been discovered that even if a Th2 response is not damaging, it is also necessary to induce a high Th1 response. Indeed, experimental results now demonstrate that a protective effect may be more easily correlated with a Th1 response than with a Th2 response.

Contrary to what was initially sought (D'Elios *et al*., J. Immunol. (1997) 158: 962), the present application therefore reveals the importance of inducing an inflammatory-type Th1 response at the time of immunization, without which a protective effect cannot be observed.

Consequently, the subject of the present invention is:

(i) The use of an immunogenic agent derived from a microorganism capable of infecting the gastroduodenal mucous membrane of a mammal, *e.g.*, derived from *Helicobacter*, in the manufacture of a pharmaceutical composition intended for the induction of a Th1-type immune response against the said microorganism, *e.g.*, *Helicobacter*, for treating or preventing an infection, *e.g.*, a *Helicobacter* infection in a mammal; and

(ii) a method for preventing or treating an infection promoted by a microorganism capable of infecting the gastroduodenal mucous membrane of a mammal, *e.g.*, a *Helicobacter* infection, according to which there is administered to

the mammal, in one or more applications, at least one immunogenic agent derived from the said microorganism, *e.g.*, from *Helicobacter*, and by which a Th1-type immune response is induced against, *e.g.*, *Helicobacter*.

The induction of a useful Th1 response can be demonstrated for the purposes of the present invention by estimating the relative level of the Th1 response relative to the Th2 response by comparing, for example, the IgG2a and IgG1 levels induced in mice against *Helicobacter*, which are respectively indicative of the coming into play of the Th1 and Th2 responses. Indeed, the Th1 response which is sought is generally accompanied by a Th2 response. However, it is considered that the Th2 response should not be significantly predominant relative to the Th1 response. The IgG2a and IgG1 levels induced in mice can be assessed conventionally using an ELISA test, provided that the tests used for each of the two subisotypes are of the same sensitivity and, in particular, that the anti-IgG2a and anti-IgG1 antibodies are of the same affinity.

The quantities of IgG2a and IgG1 can be measured in particular using an ELISA test that is identical or similar to that described below. The wells of a polycarbonate ELISA plate are coated with 100 μl of a bacterial extract from *Helicobacter, e.g.*, *H. pylori*, at about 10 μg/ml in carbonate buffer. The ELISA plate is incubated for 2 hours at 37°C and then overnight at 4°C. The plate is washed with PBS buffer (phosphate buffered saline) containing 0.05% Tween 20 (PBS/Tween buffer). The wells are saturated with 250 μl of PBS containing 1% bovine serum albumin to prevent nonspecific binding of the antibodies. After incubating for one hour at 37°C, the plate is washed with PBS/Tween buffer. The antiserum collected from mice, a number of days after the latter have received the composition intended to induce a Th1-type immune response against *Helicobacter*, is serially diluted in PBS/Tween buffer. 100 μl of the dilutions are added to the wells. The plate is incubated for 90 minutes at 37°C, washed, and evaluated according to standard procedures. For example, a goat antibody to mouse IgG2a or IgG1, coupled to an

enzyme such as peroxidase, is used. The incubation in the presence of this antibody is continued for 90 minutes at 37°C. The plate is washed and then the reaction is developed with the appropriate substrate, for example, O-phenyldiamine dihydrochloride when the enzyme used is peroxidase. The reaction is evaluated by colorimetry by measuring the absorbance by spectrophotometry. The IgG2a or IgG1 titre of the antiserum corresponds to the reciprocal of the dilution giving an absorbance of 1.5 at 490 nm.

The induction of a useful Th1 response for the purposes of the present invention is marked by a ratio of the ELISA IgG2a:IgG1 titers in mice which should be greater than 1/100, 1/50, or 1/20, advantageously greater than 1/10, preferably greater than 1/3, most preferably greater than ½, 5, or 10. When this ratio is around 1, the Th1/Th2 response is said to be mixed or balanced. When the ratio is greater than or equal to 5, the Th1 response is then said to be preponderant.

The production of a Th1 (or Th2) response in mice is predictive of a Th1 (or Th2) response in man. Although it is easier to evaluate the type of response in mice, it can also be done in man by measuring the levels of cytokines specific for the Th1 response on the one hand and, on the other hand, for the Th2 response, which are subsequently induced. The Th1 and Th2 responses can be evaluated directly in man relative to each other on the basis of the levels of cytokines specific for the two types of response (see above), *e.g.*, on the basis of the IFN-γ/IL-4 ratio.

Alternatively, if the assay method described above is used, it is possible to predict that the ELISA titre that reflects the quantity of IgG2a should be equal to or greater than 10,000, preferably equal to or greater than 100,000, in a particularly preferred manner, equal to or greater than 1,000,000; this then means that the Th1 response is significant.

The mammal for which the pharmaceutical composition or the method is intended is advantageously a primate, preferably a human.

It is possible to induce a Th1 response against *Helicobacter* by adjusting a

number of factors, such as, for example, the route of administration. It has indeed been demonstrated that by using the systemic or parenteral route, a level of protection can be obtained that is similar to or greater than that observed when the mucosal route is used.

Accordingly, the subject of the invention is in particular:

(i) the use of an immunogenic agent derived from *Helicobacter*, in the manufacture of a pharmaceutical composition intended to be administered by the systemic or parenteral route in the part of a mammal, especially a primate, situated under its diaphragm, for treating or preventing a *Helicobacter* infection; and

(ii) a method for preventing or treating a *Helicobacter* infection in a mammal, according to which there is administered to the said mammal, in one or more applications, by the systemic or parenteral route, at least one immunogenic agent derived from *Helicobacter*.

As regards the method, it is indicated that, advantageously, the administration of the immunogenic agent by the systemic or parenteral route is repeated once or several times, preferably at least twice, for the desired immune response to be induced. A preferred method by which a protective effect is obtained is in particular a method according to which the immunogenic agent is administered exclusively by the systemic or parenteral route (strict systemic route). "A method in which the administration of the immunogenic agent is carried out by the strict systemic route" is defined as a method not using a route of administration other than the systemic route. For example, a method in which the immunogenic agent is administered by the systemic route and by the mucosal route does not correspond to the definition given above. In other words, "a method in which the administration of the immunogenic agent is carried out by the strict systemic route" should be understood to mean a method in which the immunogenic agent is administered by the systemic route excluding any other route, in particular the mucosal route.

Still as regards the method, the administration by the systemic or parenteral

route is advantageously carried out in the subdiaphragmatic part of the mammal.

The immunogenic agent derived from *Helicobacter* is advantageously selected from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide and a polypeptide from *Helicobacter* in purified form. The immunogenic agent can also be a polynucleotide molecule, especially a DNA molecule including a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of elements necessary for its expression in a mammalian cell, or alternatively a viral vaccinal vector including a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of elements necessary for its expression in a mammalian cell.

For the purposes of the present invention, a preparation of inactivated bacteria can be obtained according to conventional methods well known to persons skilled in the art. Likewise for a bacterial lysate. A dose of inactivated bacteria or cell lysate, appropriate for prophylactic or therapeutic purposes, can be determined by persons skilled in the art and depends on a number of factors, such as the individual for whom the vaccine is intended, *e.g.*, the individual's age, the antigen itself, the route and mode of administration, the presence/absence or the type of adjuvant, as can be determined by persons skilled in the art. In general, it is indicated that an appropriate dose is from about 50 μg to 1 mg at about 1 mg of lysate.

A peptide or a polypeptide derived from *Helicobacter* can be purified from *Helicobacter* or obtained by genetic engineering techniques or alternatively by chemical synthesis. The latter process is advantageous in the case of peptides. "Peptide" is any amino acid chain of less than about 50 amino acids. When the size is greater, the term "polypeptide," which is also interchangeable with the term "protein," is used. A useful peptide or polypeptide for the purposes of the present invention can be identical or similar to that which exists under natural conditions. It is similar in that it is capable of inducing an immune response of the same type but it can include certain structural variations such as, for example, a mutation, the addition of a residue

of a lipid nature, or, alternatively, it can be in fusion polypeptide or peptide form.

An appropriate dose of peptide or polypeptide for prophylactic or therapeutic purposes can be determined by persons skilled in the art and depends on a number of factors, such as the individual for whom the vaccine is intended, *e.g.*, the age of the individual, the antigen itself, the route and mode of administration, the presence/absence or the type of adjuvant, as can be determined by persons skilled in the art. In general, it is indicated that an appropriate dose is from about 10 μg to about 1 mg, preferably at about 100 μg.

The DNA molecule can advantageously be a plasmid that is incapable both of replicating and of substantially integrating into the genome of a mammal. The above-mentioned coding sequence is placed under the control of a promoter allowing expression in a mammalian cell. This promoter can be ubiquitous or specific for a tissue. Among the ubiquitous promoters, there may be mentioned the Cytomegalovirus early promoter (described in U.S. Patent No. 4,168,062) and the Rous sarcoma virus promoter (described in Norton & Coffin, Molec. Cell. Biol. (1985) 5: 281). The desmin promoter (Li *et al.*, Gene (1989) 78: 244443; Li & Paulin, J. Biol. Chem. (1993) 268: 10403), which is a selective promoter, allows expression in muscle cells and also in skin cells. A promoter specific for muscle cells is, for example, the promoter of the myosin or dystrophin gene. Plasmid vectors that can be used for the purposes of the present invention are described, *i.a.*, in WO 94/21797 and Hartikka *et al.*, Human Gene Therapy (1996) 7: 1205.

In a useful pharmaceutical composition for the purposes of the present invention, the nucleotide molecule, *e.g.*, the DNA molecule, can be formulated or otherwise. The choice of formulation is highly varied. The DNA can be simply diluted in a physiologically acceptable solution with or without carrier. When the latter is present, it can be isotonic or weakly hypertonic and can have a low ionic strength. For example, these conditions can be fulfilled by a sucrose solution, *e.g.*, at 20%.

Alternatively, the polynucleotide can be combined with agents that promote entry into the cell. This can be (i) a chemical agent that modifies cell permeability, such as bupivacaine (see, for example, WO 94/16737), or (ii) an agent that is combined with the polynucleotide and that acts as a vehicle facilitating the transport of the polynucleotide. The latter may be in particular cationic polymers, *e.g.*, polylysine or a polyamine, *e.g.*, derivatives of spermine such as spermidine (see WO 93/18759). This can also be fusogenic peptides, *e.g.*, GALA or Gramicidin S (see WO 93/19768) or, alternatively, peptides derived from viral fusion proteins.

This can also be anionic or cationic lipids. The anionic or neutral lipids have been known for a long time to be capable of serving as transporting agents, for example, in the form of liposomes, for a large number of compounds, including polynucleotides. A detailed description of these liposomes, of their constituents, and of the processes for their manufacture is, for example, provided by *Liposomes: A Practical Approach*, RPC New Ed., IRL press (1990).

~~The cationic lipids are also known and are commonly used as transporting~~ agents for polynucleotides. There may be mentioned for example Lipofectin™ also known by the name DOTMA (N-[1-(2,3-dioleyloxy) propyl]-N,N,N-trimethylammonium chloride), DOTAP (1,2-bis(oleyloxy)-3-(trimethyl-ammonio)propane), DDAB (dimethyldioctadecylammonium bromide), DOGS (dioctadecylamidoglycyl spermine), and cholesterol derivatives, such as DC-chol (3-beta-(N-(N',N'-dimethylaminoethane) carbamoyl) cholesterol). A description of these lipids is provided by EP 187,702, WO 90/11092, U.S. Patent No. 5,283,185, WO 91/15501, WO 95/26356, and U.S. Patent No. 5,527,928. The cationic lipids are preferably used with a neutral lipid such as DOPE (dioleylphosphatidylethanolamine) ~~as is, for example, described in WO 90/11092~~.

Gold or tungsten microparticles can also be used as transporting agents, as described in WO 91/359, WO 93/17706, and Tang *et al.*, Nature (1992) <u>356</u>: 152. In this particular case, the polynucleotide is precipitated on the microparticles in the

presence of calcium chloride and spermidine, and then the whole is administered by a high-speed jet into the dermis or into the epidermis using an apparatus with no needle, such as those described in U.S. Patent Nos. 4,945,050 and 5,015,580, and WO 94/24243.

The quantity of DNA that can be used to vaccinate an individual depends on a number of factors such as, for example, the strength of the promoter used to express the antigen, the immunogenicity of the product expressed, the condition of the mammal for whom the administration is intended (*e.g.*, the weight, age, and general state of health), the mode of administration, and the type of formulation. It is indicated in particular that the administration by the intramuscular route requires a larger quantity of DNA than the administration by the intradermal route using an apparatus with no needle. In general, an appropriate dose for prophylactic or therapeutic use in an adult of the human species is from about 1 μg to about 5 mg, preferably from about 10 μg to about 1 mg, most preferably from about 25 μg to about 500 μg.

Vaccinal vectors are among the immunogenic agents mentioned above. Adenoviruses and poxviruses in particular are among the vectors of viral origin. An example of a vector derived from an adenovirus, as well as a method for constructing a vector capable of expressing a DNA molecule encoding a useful peptide or polypeptide for the purposes of the present invention, are described in U.S. Patent No. 4,920,209. Poxviruses that can be used likewise are, for example, the vaccinia and canarypox viruses. They are described respectively in U.S. Patents Nos. 4,722,848 and 5,364,773 (see also, *e.g.*, Tartaglia *et al.*, Virology (1992) 188: 217 and Taylor *et al.*, Vaccine (1995) 13: 539). Poxviruses capable of expressing a useful peptide or polypeptide for the purposes of the present invention can be obtained by homologous recombination, as described in Kieny *et al.*, Nature (1984) 312: 163, such that the DNA fragment encoding the peptide or polypeptide is placed under conditions appropriate for its expression in mammalian cells. A bacterial vector such as the bile

Calmette-Guérin bacillus can also be used.

In general, the dose of a viral vector intended for prophylactic or therapeutic purposes can be from about 1×10^4 to about 1×10^{11}, advantageously from about 1×10^7 to about 1×10^{10}, and preferably from about 1×10^7 to about 1×10^9 plaque forming units *per* kilogram.

The immunogenic agent derived from *Helicobacter* can be any polypeptide from *Helicobacter*, *e.g.*, *H. pylori*. This can be in particular a polypeptide present in the cytoplasm, a polypeptide of the inner or outer membrane, or a polypeptide secreted in the external medium. Numerous polypeptides from *Helicobacter* have already been described in the literature, either with reference to their amino acid sequence deduced from the sequence of the cloned or identified corresponding gene, or with reference to a purification process that makes it possible to obtain them in a form isolated from the rest of their natural environment. As a guide, the following documents are mentioned in particular: WO 94/26901 and WO 96/34624 (HspA), WO 94/09023 (CagA), WO 96/38475 (HpaA), WO 93/181150 (cytotoxine), WO 95/27506 and Hazell *et al.*, J. Gen. Microbiol. (1991) <u>137</u>: 57 (catalase), FR 2 724 936 (membrane receptor for human lactoferrin), WO 96/41880 (AlpA), EP 752 473 (FibA) and O'Toole *et al.*, J. Bact. (1991) <u>173</u>: 505 (TsaA). Other polypeptides are also described in WO 96/40893, WO 96/33274, WO 96/25430, and WO 96/33220. A useful polypeptide for the purposes of the present invention can be identical or similar to one of those cited as a reference insofar as it is capable of promoting an immune response against *Helicobacter*. In order to meet this last condition, the immunogenic agent can also be a peptide derived from a polypeptide cited as a reference.

Advantageously, a polypeptide selected from the UreA and UreB subunits of *Helicobacter* urease is used (see WO 90/4030). Preferably, both are used, combined in urease apoenzyme form or alternatively in multimeric form (see WO 96/33732).

Likewise, a useful vaccinal vector or DNA molecule for the purposes of the present invention includes a sequence that can encode any polypeptide or peptide

described above.

A DNA molecule, or preferably a viral vaccinal vector, can also include a sequence encoding a cytokine, for example, a lymphokine, such as interleukin-2 or interleukin-12, under the control of elements appropriate for expression in a mammalian cell. An alternative to this option also consists in adding to a useful pharmaceutical composition for the purposes of the present invention comprising a DNA molecule or a vector, another molecule, or viral vector encoding a cytokine.

A useful pharmaceutical composition for the purposes of the present invention can contain a single immunogenic agent or several. For example, an advantageous composition can comprise UreA and UreB, *e.g.*, in apoenzyme form, as well as one or more other polypeptides selected in particular from those mentioned above. Likewise, when a DNA molecule or a vaccinal vector is involved, the composition can contain several of them, each encoding a particular polypeptide or a single DNA molecule or vaccinal vector encoding several peptides or polypeptides.

A useful pharmaceutical composition for the purposes of the present invention can, in addition, contain compounds other than the immunogenic agent itself, the nature of these compounds depending, to a certain extent, on the nature of the immunogenic agent, inactivated bacteria, cell lysate, peptide, or polypeptide, DNA molecule, or vaccinal vector. Thus, as has already been seen above, when a DNA molecule is involved, the pharmaceutical composition can include various formulation agents. A composition can also include an appropriate adjuvant for administration by the systemic or parenteral route, *e.g.*, an aluminum compound, such as aluminum hydroxide, aluminum phosphate, or aluminum hydroxyphosphate. In general, it is indicated that inactivated bacteria may not require the addition of an adjuvant. The same is true as regards the DNA molecules. On the other hand, the presence of an adjuvant is preferable when the immunogenic agent is a bacterial lysate or a purified peptide or polypeptide. Finally, when the immunogenic agent is a vaccinal vector, the use thereof is preferably avoided so that the immune response towards the vector itself

remains minimal.

In addition to the aluminum compounds, a large number of appropriate adjuvants for administration by the systemic or parenteral route exist in the state of the art among which persons skilled in the art are capable of selecting the one that best corresponds to their needs; in particular a compound capable of promoting the induction of a Th1-type immune response or a balanced response of the Th1 + Th2 type. As a guide, there can be mentioned in particular liposomes; ISCOMS; microspheres; protein chochleates; vesicles consisting of nonionic surfactants; cationic amphiphilic dispersions in water; oil/water emulsions; muramidyldipeptide (MDP) and its derivatives such as glucosyl muramidyldipeptide (GMDP), threonyl-MDP, murametide and murapalmitin; and QuilA and its subfractions; as well as various other compounds such as monophosphoryl-lipid A (MPLA) major lipopolysaccharide from the wall of a bacterium, for example of *E. coli*, *Salmonella minnesota*, *Salmonella typhimurium*, or *Shigella flexneri*; algan-glucan; gamma-inulin; calcitriol; and loxoribine.

Sub C2 ~~Useful liposomes for the purposes of the present invention can be selected in~~ particular from pH-sensitive liposomes, such as those formed by mixing cholesterol hemisuccinate (CHEMS) and dioleyl phosphatidyl ethanolamine (DOPE); liposomes containing cationic lipids recognized for their fusiogenic properties, such as 3-beta-(N-(N',N'-dimethylamino-ethane)carbamoyl)cholesterol (DC-chol) and its equivalents, which are described in U.S. Patent No. 5,283,185 and WO 96/14831, dimethyldioctadecylammonium bromide (DDAB) and the BAY compounds described in EP 91645 and EP 206 037, for example Bay R1005 (N-(2-deoxy-2-L-leucylamino-beta-D-glucopyranosyl)-N-octa-decyldodecanoylamide acetate; and liposomes containing MTP-PE, a lipophilic derivative of MDP (muramidyldipeptide). These ~~liposomes are useful~~ for adding as adjuvant to all the immuno~~genic agents cited.~~

Sub C3 ~~Useful ISCOMs for the purposes of the present invention can be selected in~~ particular from those compounds of QuilA or of QS-21 combined with cholesterol and

optionally also with a phospholipid such as phosphatidylcholine. These are particularly advantageous for the formulation of the lipid-containing antigens.

Useful microspheres for the purposes of the present invention can be formed in particular from compounds such as polylactide-co-glycolide (PLAGA), alginate, chitosan, polyphosphazene, and numerous other polymers.

Useful protein chochleates for the purposes of the present invention can be selected in particular from those formed from cholesterol and optionally an additional phospholipid, such as phosphatidylcholine. These are especially advantageous for the formulation of the lipid-containing antigens.

Useful vesicles consisting of nonionic surfactants for the purposes of the present invention can be in particular formed by a mixture of 1-mono-palmitoyl glycerol, cholesterol, and dicetylphosphate. They are an alternative to the conventional liposomes and can be used for the formulation of all the immunogenic agents cited.

Useful oil/water emulsions for the purposes of the present invention can be selected in particular from MF59 (Biocine-Chiron), SAF1 (Syntex), and the montanides ISA51 and ISA720 (Seppic).

A useful adjuvant for the purposes of the present invention can also be a fraction derived from the bark of the South American tree *Quillaja Saponaria Molina*; for example, QS-21, a fraction purified by HPLC chromatography as is described in U.S. Patent No. 5,057,540. Since some toxicity may be associated with QS-21, it may be advantageous to use the latter in liposomes especially based on sterol, as is described in WO 96/33739.

Finally, an adjuvant effect can also be obtained by adding lipid to the useful peptide or polypeptide for the purposes of the present invention. The combination, by covalent bonding, of such a peptide or polypeptide with a lipid or a lipid-containing compound capable of promoting the induction of a Th1-type immune response, so as to form a lipid-containing lipopeptide or polypeptide conjugate, can be achieved in

various ways known to persons skilled in the art. For example, it is possible to use one of the compounds described in EP 431 327 such as N-palmitoyl-S-2,3-(bispalmitoyloxy) propylcysteinylseryl serine (Pam_3CSS), which is coupled by known processes to the N-terminal end of the peptide or polypeptide.

The therapeutic or prophylactic efficacy of a method or of a use according to the invention can be evaluated according to standard methods, *e.g.*, by measuring the induction of an immune response or the induction of a therapeutic or protective immunity using, *e.g.*, the mouse/*H. felis* model and the procedures described in Lee *et al.*, Eur. J. Gastroenterology & Hepatology (1995) 7: 303 or Lee *et al.*, J. Infect. Dis. (1995) 172: 161. Persons skilled in the art will realize that *H. felis* can be replaced in the mouse model by another *Helicobacter* species. For example, the efficacy of an immunogenic agent derived from *H. pylori* is preferably evaluated in a mouse model using an *H. pylori* strain adapted to mice. The efficacy can be determined by comparing the level of infection in the gastric tissue (by measuring the urease activity, the bacterial load, or the condition of the gastritis) with that in a control group. A therapeutic effect or a protective effect exists when the infection is reduced compared with the control group.

A useful pharmaceutical composition for the purposes of the present invention can be manufactured in a conventional manner. In particular, it can be formulated with a pharmaceutically acceptable carrier or diluent, *e.g.*, water or a saline solution. In general, the diluent or carrier can be selected according to the mode and route of administration and according to standard pharmaceutical practices. Appropriate carriers or diluents, as well as what is essential for the preparation of a pharmaceutical composition, are described in *Remington's Pharmaceutical Sciences*, a standard reference book in this field.

The methods according to the invention, as well as the compositions useful for these purposes, can be used to treat or prevent, *i.a.*, *Helicobacter* infections and consequently the gastroduodenal diseases associated with these infections, including

acute, chronic, or atrophic gastritis, and peptic ulcers, *e.g.*, gastric or duodenal ulcers.

The systemic route that is used can be the parenteral route, which can itself be chosen from the intravenous, intramuscular, intradermal, intraepidermal, and subcutaneous routes; the latter four being however preferred to the intravenous route. The intramuscular and subcutaneous routes are particularly recommended. In all cases, the use that will be made of the pharmaceutical composition can call into play a site of administration situated under the diaphragm of an individual. The dorsolumbar region constitutes, for example, an appropriate site of administration.

To obtain a protective or therapeutic effect, the operation that consists of administering, for example, by the subdiaphragmatic systemic route, a useful pharmaceutical composition for the purposes of the present invention can be repeated once or several times, leaving a certain time interval between each administration; which interval is of the order of a week or a month. Its precise determination is within the capability of persons skilled in the art and can vary according to various factors, such as the nature of the immunogenic agent, the age of the individual, and the like. In this particular case, the administration is said to be of the strict systemic type. By way of a nonlimiting illustration, there may be mentioned a vaccination scheme that consists of administering the urease apoenzyme three times by the subcutaneous route, in the dorsolumbar region, with an interval of two to four weeks between each administration.

According to an alternative mode, it is possible to envisage operating in a strict systemic mode of administration, but using immunogenic agents that vary during the administrations constituting the steps of the vaccination procedure. By way of a nonlimiting illustration, there may be mentioned a vaccination scheme by the strict systemic route, in three steps: a first administration (priming) consists of administering a pox vector encoding, *e.g.*, UreA and UreB, followed by two consecutive administrations (boosters) of the urease apoenzyme.

In general, the subject of the invention is therefore also a pharmaceutical

composition intended to treat or prevent a *Helicobacter* infection which includes, for consecutive administration, several products, each of the products being formulated so as to be administered by the subdiaphragmatic systemic route and containing an immunogenic agent derived from *Helicobacter* selected independently from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide, a polypeptide from *Helicobacter* in purified form, a DNA molecule comprising a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression, and a vaccinal vector including a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression, preferably provided that when a first product contains a peptide or a polypeptide and a second product contains a DNA molecule or a vaccinal vector, the coding sequence of the DNA molecule or of the vaccinal vector encodes the peptide or polypeptide contained in the first product.

Finally, an alternative vaccination procedure comprising several administrations staggered over time, *e.g.*, within time intervals of the order of a week or a month, to be determined by persons skilled in the art, can include a first administration by the subdiaphragmatic systemic route and a second administration by the mucosal route other than the intranasal route, *e.g.*, by the ocular, oral, *e.g.*, buccal or gastric, pulmonary, intestinal, rectal, vaginal, or urinary route. By way of a nonlimiting illustration, there can be mentioned a vaccination procedure that consists of administering a DNA molecule or a vaccinal vector by the subdiaphragmatic systemic route and then in administering a polypeptide by the gastric route, the DNA molecule or the vaccinal vector preferably encoding the polypeptide administered by the gastric route.

In general, the subject of the invention is therefore also a pharmaceutical composition intended to treat or prevent a *Helicobacter* infection that contains, for consecutive administration, several products; one of the products being formulated so as to be administered by the subdiaphragmatic systemic route and another product

being formulated so as to be administered by a mucosal route other than the intranasal route; each of the products containing an immunogenic agent derived from *Helicobacter* selected independently from a preparation of inactivated *Helicobacter* bacteria, a *Helicobacter* cell lysate, a peptide, a polypeptide from *Helicobacter* in purified form, a DNA molecule including a sequence encoding a peptide, or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression and a vaccinal vector including a sequence encoding a peptide or a polypeptide from *Helicobacter* placed under the control of the elements necessary for its expression, preferably provided that when a first product contains a peptide or a polypeptide and a second product contains a DNA molecule or a vaccinal vector, the coding sequence of the DNA molecule or of the vaccinal vector encodes the peptide or polypeptide contained in the first product.

A vaccinal vector contained in a product intended to be administered by the mucosal route can be chosen from those described above. In addition, it can be selected from bacterial vectors such as *Shigella, Salmonella, Vibrio cholerae, Lactobacillus*, and *Streptococcus*.

Nontoxic mutant strains of *Vibrio cholerae* that can be useful as live vaccine vectors are described, for example, in Mekalanos *et al.*, Nature (1983) 306: 551 and U.S. Patent No. 4,882,278 (strain in which a substantial part of the region encoding each of the two alleles *ctxA* has been deleted so that no functional toxin can be produced); WO 92/11354 (strain in which the *irgA* locus is inactivated by mutation; this mutation may be combined in the same strain with *ctxA* mutations); and WO 94/1533 (mutant obtained by deletion lacking functional *ctxA* and *attRS1* sequences). These strains can be modified genetically to express heterologous antigens as described in WO 94/19482.

Attenuated strains of *Salmonella typhimurium*, genetically modified or otherwise for the recombinant expression of heterologous antigens, as well as their use as vaccines, are described in Nakayama *et al.*, BioTechnology (1988) 6: 693 and WO

92/11361.

Other bacteria useful as vaccinal vectors are described in High *et al.*, EMBO (1992) 11: 1991 and Sizemore *et al.*, Science (1995) 270: 299 (*Shigella flexneri*); Medaglini *et al.*, Proc. Natl. Acad. Sci. USA (1995) 92: 6868 (*Streptococcus gordonii*); and Flynn J.L., Cell. Mol. Biol. (1994) 40 (suppl. I): 31, WO 88/6626, WO 90/0594, WO 91/13157, WO 92/1796, and WO 92/21376 (Calmette-Guérin bacillus).

In bacterial vectors, the DNA sequence encoding a peptide or polypeptide from *Helicobacter* can be inserted into the bacterial genome or alternatively remain in the free state, carried by a plasmid. Obviously, this sequence is placed under the control of the elements necessary for its expression in the bacterial vector.

These bacterial vectors for administration by the mucosal route can be used in combination with an appropriate adjuvant. Such adjuvants may be chosen from bacterial toxins, *e.g.*, the cholera toxin (CT), the *E. coli* heat-labile toxin (LT), the *Clostridium difficile* toxin, and the *Pertussis* toxin (PT), or combinations, subunits, toxoids, or mutants that are derived therefrom. For example, it is possible to use a purified preparation of the native cholera toxin B subunit (CTB). Fragments, homologues, derivatives, and fusions of these toxins are equally suitable provided they retain the adjuvant activity. Preferably, a mutant is used whose toxicity is reduced. Such mutants are described in, *e.g.*, WO 95/17211 (mutant CT Arg-7-Lys), WO 96/6627 (mutant LT Arg-192-Gly), and WO 95/34323 (mutant PT Arg-9-Lys and Glu-129-Gly). Other LT mutants that can also be used carry at least one of the following mutations: Ser-63-Lys, Ala-69-Gly, Glu-110-Asp, and Glu-112-Asp.

Other compounds, such as MPL~~A, PLGA, DC-chol~~, and QS-21 can also be used ~~as adjuvants~~ for the mucosal route.

The invention also includes immunization methods for treating or preventing *Helicobacter* (*e.g.*, *H. pylori*) infection that involve mucosal (*e.g.*, oral, intranasal, intragastric, pulmonary, intestinal, rectal, ocular, vaginal, or urinary tract) administration, followed by parenteral (*e.g.*, intramuscular, subcutaneous, intradermal,

intramuscular, intravenous, or intraperitoneal). In one example of these methods, mucosal administration is carried out to prime an immune response to an antigen, and parenteral administration is carried out to boost the immune response to the antigen. Other examples of these methods involve alternating parenteral and mucosal administrations, for example, the following pattern can be used: intramuscular administration, combined intragastric + intranasal administration, intramuscular administration, and combined intragastric + intranasal administration. Antigens, formulations, adjuvants, administration regimens, specific mucosal and parenteral routes, and dosages to be used can readily be determined by one skilled in the art. Specific examples of these parameters that can be adapted for use in these methods are provided above.

In the description above, reference was made essentially to *Helicobacter* infections and to the means for combating them by way of prevention and prophylaxis. However, it should be understood that the principles and methods stated above can be applied *mutatis mutandis* to any other infection induced by any microorganism whose seat is the stomach, the duodenum or the intestine.

It is specified, in addition, that all the documents published and cited in the present application are incorporated by reference.

The invention is illustrated below with reference to the following figures.

ins B1 Figure 1 refers to Example 1 and presents a study of the local response in the salivary glands (Figure 1A) and in the stomach (Figure 1B) evaluated by ELISPOT by measuring the quantity of anti-urease IgA induced, expressed as spots/10^6 cells (Figure 1A) or as number of responding mice, exhibiting more than 2 IgA spots/mouse, (Figure 1B), after (a) administration of urease at D0 by the subcutaneous route (SC) in the left posterior sublumbar part [(a) and (c)] or in the neck [(b) and (d)], followed by a booster by the nasal route (N) and intragastric route (IG), at D28 [(a) and (b)] or at D28 and D56 [(c) and (d)].

ins B2 ~~Figure 2 refers to Example 1 and presents the levels of urease activity after a~~

challenge, measured 4 hours after sacrificing mice which have received 3 times, on D0, D28 and D56, an inactivated bacterial preparation by the intragastric route [(a) and (c)] or subcutaneous route in the left posterior sublumbar part (b). In experiment (c), 10 μg of cholera toxin were added to the bacterial preparation. Experiments (d) ~~and (e) correspond respectively to the positive and negative controls~~.

ins C6

~~Figure 3 refers to Example~~ 1 and ~~presents the~~ levels of urease ~~activity after~~ a challenge, measured 4 hours after sacrificing mice which have received 3 times, on D0, D28, and D56: (a) a urease preparation encapsulated at about 80% in DC-chol liposomes, in the dorsolumbar muscles; or (b) a urease preparation with cholera toxin adjuvant, by the intragastric route. Experiments (c) and (d) correspond respectively to the positive and negative controls.

ins C7

~~Figure 4 refers to Example 1 and presents~~ the ~~levels of urease activity~~ after a challenge measured 4 hours after sacrificing mice which have received 3 times, on D0, D28, and D56: (a) a urease preparation with cholera toxin adjuvant, by the intragastric route or (b) a urease preparation with QS-21 adjuvant, by the subcutaneous route in the left posterior sublumbar part. Experiments (c) and (d) correspond respectively to the positive and negative controls.

ins B3

~~Figure 5 presents the quantities of serum immunoglobulins induced in monkeys~~ subjected to the immunization procedures described in Example 2, and expressed as ELISA titre. A control group comprising 4 monkeys and three test groups are formed, each of the test groups comprising 8 monkeys; each test group is divided into two subgroups of 4 monkeys, one receiving only the inactivated *H. pylori* preparation (1, 2, and 3) and the other receiving the inactivated *H. pylori* preparation and recombinant urease (1u, 2u, and 3u). Group 1 and 1u corresponds to the administration procedure [nasal + intragastric, 4 times]; group 2 and 2u corresponds to the administration procedure [intramuscular, 4 times]; group 1 and 1u corresponds to the administration procedure [nasal + intragastric, intramuscular, nasal + intragastric, intramuscular]. The ELISA titre is measured three times: a first time at D0 (white band), a second

time at D42 ~~(shaded band), a third time at D78 (black band).~~

Figures 6A and 6B ~~show the urease activity~~ (Figure 6A) ~~measured after 4 hours~~ (OD_{550} nm) using the Jatrox test (Procter & Gamble) and the bacterial load in mice infected with *H. pylori* and then submitted to various treatments A - H [A: LT + urease, orally; B: QS-21 + urease, parenterally in the neck; C: QS-21 + urease, parenterally in the lumbar region; D: QS-21 alone, sub-cutaneously in the lumbar region; E: Bay R1005 + urease, parenterally in the neck; F: Bay R1005 + urease, parenterally in the lumbar region; G: Bay R1005 alone, sub-cutaneously in the lumbar region (control); H: saline, sub-cutaneously in the lumbar region (positive control)]. I ~~represents the negative control.~~

Figure 7 presents the results of immunization of mice with a mucosal prime/parenteral boost strategy with urease induced the most efficacious protection against challenge with *H. pylori*. Mice were immunized either orally with 25 μg urease + 5 μg LT or parenterally with 10 μg urease with or without 100 μg alum adjuvant. The mice were primed with orally administered urease + LT, 2 booster doses were administered three weeks apart by either the parenteral or oral route, as shown in the figure. Mice were challenged with *H. pylori* two weeks after the last immunization and euthanized 2 weeks after challenge. At necropsy, one-third of the stomach, dissected longitudinally, was homogenized and cultured for *H. pylori*.

Figure 8 ~~shows the effect of urease immunization on experimental challenge~~ of rhesus monkeys with *H. pylori*. Monkeys were immunized with urease by parenteral routes (100 μg urease + 1 mg alum or 800 μg Bay) or by a mucosal prime (orally administered 4 mg urease +100 μg LT)/parenteral boost (urease + alum) strategy with 3 doses administered every 3 weeks followed by a fourth dose administered 20 weeks after the first priming dose. Monkeys were challenged one week after the last booster dose. The monkeys were euthanized 10 weeks after challenge, 10 punch biopsies per animal were harvested from the stomach and cultured to determine *H. pylori* colonization. Each symbol above represents the mean CFU of 10 sites cultured per

monkey. The line represents the median CFU for the treatment group.

Figure 9 presents gastritis scores in immunized and unimmunized rhesus monkeys following challenge with *H. pylori*. Monkeys were orally immunized with a priming dose of 4 mg urease + 100 μg LT followed 3 weeks later with 2 parenteral administered 20 weeks after the first priming dose. Monkeys were challenged one week after the last booster dose. The monkeys were euthanized 10 weeks after challenge, 2 cm^2 sections were taken from the corpus, antrum and corporal-antral junction, fixed in 10% buffered formalin, embedded in paraffin and sections stained with H & E. Gastritis, typified by infiltration of lymphocytes, plasma cells, and polymorphonuclear cells, was scored by microscopic examination of stained sections. Each symbol above represents the mean gastritis score of the 3 regions from each monkey.

Figure 10 presents epithelial changes in immunized and unimmunized rhesus monkeys following challenge with *H. pylori*. Monkeys were orally immunized with a priming dose of 4 mg urease + 100 μg LT followed 3 weeks later with 2 parenteral doses of 100 μg urease + 1 mg alum every 3 weeks and 1 parenteral dose of urease + alum administered 20 weeks after the first priming dose. Monkeys were challenged one week after the last booster dose. The monkeys were euthanized 10 weeks after challenge, 2 cm^2 sections were taken from the corpus, antrum and corporal-antral junction, fixed in 10% buffered formalin, embedded in paraffin and sections stained with H & E. Epithelial changes, defined as metaplasia, atrophy and/or hyperplasia, was scored by microscopic examination of stained sections. Each symbol above represents the mean gastritis score of the 3 regions from each monkey.

Example 1: Immunization studies in mice

1A - Materials and methods

Mice

6/8-week old female Swiss mice were provided by Janvier (France). During

the whole experiment, sterilized materials were used; the cages were protected by "isocaps;" the mice were fed with filtered water and irradiated food.

Administration procedure

During each experiment, the mice received 3 doses of the same product; each dose at 28-day intervals (days 0, 28, and 56). The administration of the product was carried out by the nasal route (up to 50 μl on waking mice), by the oral route (300 μl in 0.2 M $NaHCO_3$ by gastric gavage), or by the subcutaneous route (300 μl under the skin of the neck or under the skin on the left side of the lumbar region). In some cases, an intramuscular inoculation was carried out (50 μl) in the dorsolumbar muscles of anaesthetized mice. Ten μg of urease were administered by the nasal, subcutaneous or intramuscular route, and 40 μg by the oral route. As regards the inactivated bacterial preparation, 400 μg of cells were administered by the subcutaneous route or by the oral route.

Antigens and adjuvants

The *H. pylori* urease apoenzyme was expressed in *E. coli* and purified as has been described in Example 5 of WO 96/31235. In the remainder of the text, the simple term of urease is used to designate this apoenzyme.

A preparation of inactivated *H. pylori* bacteria (WC) was prepared as follows: a bottle of frozen bacteria ATCC 43579 is diluted in a two-phase medium in a 75 cm^2 flask (Costar). This medium is composed of a solid constituent (10 ml Columbia agar (BioMérieux) + 6% sheep blood (BioMérieux)) and a liquid constituent (3 ml of TSB, BioMérieux). The flask is placed in a generbag containing a microaer (BioMérieux) and incubated with gentle shaking for 48 hours at 37°C. Culture is then analyzed (mobility, urease, catalase, and production of oxidase) and centrifuged (optionally after having grouped together several flasks) at 3,000 rpm for 20 minutes at 4°C. The pellet is resuspended in PBS (BioMérieux) containing 1% formalin (37% formalin,

Sigma). The volume is adjusted so as to obtain a final concentration of 2 mg/ml (1 ml having an OD of 1 at 600 nm before centrifugation corresponds to 377 μg of protein). The product is mixed gently at 4°C for 4 hours, washed 3 times in PBS, and the final solution is concentrated to 100 μg of protein/50 μl. The aliquots are kept at -70°C.

~~DC-chol liposomes containing urease~~ are prepared as follows: ~~first of all, to~~ obtain a dry lipid film containing 100 mg of DC-chol (R-Gene Therapeutics) and 100 mg of DOPC (dioleylphosphatidylcholine) (Avanti Polar Lipids), these products are mixed in powdered form in about 5 ml of chloroform. The solution is allowed to evaporate under vacuum using a rotary evaporator. The film thus obtained on the walls of the container is dried under high vacuum for at least 4 hours. In parallel, 20 mg of a urease lyophilisate and 100 mg of sucrose are diluted in 13.33 ml of 20 mM Hepes buffer pH 7.2. Ten ml of this preparation (which contains 1.5 mg of urease and 0.75% sucrose) is filtered on the 0.220 μm Millex filter and then used to rehydrate the lipid film. The suspension is stirred for 4 hours and then either extruded (10 passes on a 0.2 μm polycarbonate membrane) or microfluidized (10 passes at a pressure of 500 kPa in a Microfluidics Co Y10 microfluidizer). In the liposome suspension thus obtained, the level of encapsulated urease is from 10 to 60%. This suspension is lyophilized after having adjusted the sucrose concentration to 5% (425 mg of sucrose are added per 10 ml). Before use, the lyophilisate is taken up in an appropriate volume of water or buffer and the suspension is purified on a discontinuous sucrose gradient (steps of 0, 30, and 60%) so as to obtain a preparation in which the quantity of encapsulated urease is greater than about 70% compared with ~~the total quantity of urease~~.

Cholera toxin is ~~used as~~ mucosal adjuvant in an amount of 10 μg/dose of urease or of ~~bacterial~~ preparation.

The QS-21 (Cambridge Biosciences; Aquila) is used as adjuvant in an amount of 15 μg/dose of urease.

Challenge

Two weeks after the second booster, the mice were subjected to a gastric gavage with 300 μl of a suspension of a strain of *H. pylori* adapted to the mice, the strain ORV2002 (1×10^7 live bacteria in 200 μl of PBS; OD_{550} of about 0.5). One group which received no dose of antigen and which serves as control is challenged likewise.

Analysis of the challenge

Four weeks after the challenge, the mice were sacrificed by breaking the cervical vertebrae. The stomachs were removed in order to evaluate the urease activity and to make histological analyses. The urease activity was evaluated after 4 and 24 hours (OD at 550 nm) with the Jatrox test, Procter & Gamble) and after 24 hours the number of mice still negative was noted.

Measurement of the local antibody response by ELISPOT (salivary glands and stomach)

The ELISPOTs were performed in accordance with Mega *et al.*, J. Immunol. (1992) 148: 2030. The plates were coated with an extract of *H. pylori* proteins at a concentration of 50 μg/ml.

To test the antibody response at the level of the stomach, we modified the method as follows: half of the stomach was cut into 1-mm^2 pieces with an automatic apparatus for cutting human tissues (McIllwain Laboratories, Gilford, UK) and the digestion carried out with Dispase (2 mg/ml, Boehringer Mannheim) in 2 ml of a modified Joklil solution to which 10% horse serum (Gibco), glutamine and antibiotics were added. Four half-hour digestions were performed at 37°C with gentle mixing. The cells thus digested were filtered after each step using 70 μm filters (Falcon), and then washed 3 times in a solution of RPMI 1640 (Gibco) supplemented with 5% fetal calf serum (FCS), and incubated in the same solution for at least 4 hours in plates

covered with nitrocellulose (Millipore) (100 μl/well, 4 wells). Between 1 and 3 × 10^5 cells are obtained per half stomach (the cells of large size and the macrophages were not counted).

The biotinylated IgA and the streptavidin-biotinylated peroxidase complex were obtained from Amersham. The spots were revealed under the action of the AEC substrate (Sigma) and as soon as the plates are dry, they were counted under a microscope (magnification ×16 or ×40). The mean values corresponding to the number of IgA spots in four wells were calculated and expressed as the number of spots/10^6 cells.

Analysis of the response by ELISA

The analyses by ELISA were performed in accordance with the standard procedure (the biotinylated conjugates and the streptavidin-peroxidase were obtained from Amersham and the OPD (O-phenyl-diamine dihydrochloride) substrate from Sigma). The plates were coated with *H. pylori* extracts (5 μg/ml) in carbonate buffer. A control serum from mice directed against the *H. pylori* extract was introduced in each experiment. The titre corresponds to the reciprocal of the dilution giving an OD of 1.5 at 490 nm.

1B - Results

The results are presented in Figures 1 to 4 described above and by the following comments:

Figure 1 shows that when the subcutaneous route is used, much better results are obtained in terms of the local response both in the salivary glands and in the stomach if the administration took place in the posterior part of the mice, that is to say in the sublumbar region.

Before any comment on the subject of Figures 2 to 4, it should be noted that these figures present the results obtained with the antigen used in the form with

cholera toxin adjuvant and administered by the intragastric route. This experiment is termed standard reference experiment since the prior art CT/IG combination is that which gives the best results up until now.

Figure 2 compares the results obtained with a preparation of inactivated bacteria without adjuvant, by the intragastric route and subcutaneous route. It is clear that much better results are obtained when the subcutaneous route is used while targeting the sublumbar region. Furthermore, the results obtained after administration by the subcutaneous route are identical to, if not slightly better than, those which are obtained in the standard reference experiment with the same preparation, this time with the cholera toxin adjuvant and administered by the intragastric route.

Furthermore, reference can be made to experiments (a) to (e) the results of which in terms of urease activity 4 hours after the mice have been sacrificed are reported in Figure 2 and it is indicated that the number of mice which are still negative for the urease activity 24 hours after having been sacrificed is respectively (a) 0/8, (b) 4/8, (c) 4/8, (d) 0/8, and (e) 10/10. This is in agreement with what was previously concluded in the paragraph; namely that experiment (b) leads to results similar to those obtained during the standard reference experiment.

Fi~~gure 3 shows that a urease preparation encapsulated into DC-chol lipo~~somes and administered by the subcutaneous ~~route in the sub~~lumbar region gives results as ~~good as those obtained in the standard reference exper~~iment.

Furthermore, reference can be made to experiments (a) to (d) whose results in terms of urease activity 4 hours after the mice have been sacrificed are reported in Figure 3 and it is indicated that the number of mice which are still negative for the urease activity 24 hours after having been sacrificed is respectively (a) 5/10, (b) 4/10, (c) 0/10, and (d) 10/10. This is in agreement with what was concluded in the preceding paragraph; namely that experiment (a) leads to results similar to those obtained during the standard reference experiment.

Figure 4 shows that a urease preparation with QS-21 adjuvant and administered

by the subcutaneous route in the sublumbar region gives results as good as those obtained in the standard reference experiment.

Furthermore, reference can be made to experiments (a) to (d) whose results in terms of urease activity 4 hours after the mice have been sacrificed are reported in Figure 4 and it is indicated that the number of mice which are still negative for the urease activity 24 hours after having been sacrificed is respectively (a) 1/8, (b) 5/8, (c) 0/8, and (d) 10/10. This is in agreement with what was concluded in the preceding paragraph; namely that experiment (b) leads to results similar to those obtained during the standard reference experiment.

The table below presents the quantities of IgA, IgG1, and IgG2a induced during experiments whose results in terms of urease activity are reported in Figures 2 to 4 as well as the number of mice whose urease activity is characterized by an OD of less than 0.1 after 4 and 24 hours after sacrifice. The quantities of IgA, IgG1, and IgG2a are expressed as ELISA titre.

	urease[1] CT IG	WC IG	WC CT IG	WC SC	urease lipo DC-chol SC	urease QS-21 SC
IgA	45	91	107	63	0	1
IgG1	65700	1920	349	1273146	620000	2970399
IgG2a	20200	399	3440	42900	321000	1136095
OD < 0.1 4 hours	5/10	0/8	5/8	6/8	5/10	6/8
OD < 0.1 24 hours	4/10	0/8	4/8	4/8	5/10	5/8

Example 2: Immunization studies in monkeys

2A - Materials and methods

Monkeys

Twenty eight 2-year old monkeys (*Macaca fascicularis*) obtained from Mauritius were used in this study. Before subjecting the monkeys to the various immunization procedures described below, a biopsy showed that most of them were

chronically infected with organisms similar to *Gastrospirillum hominis* (GHLO) or *H. heilmanii*.

Administration procedures

Since nearly all the monkeys were infected with GHLOs, it was decided to test the efficacy of the various procedures in therapy. Three procedures were used, as summarized in the table below:

Group	D0	D21	D42	D63
1 and 1u	IN + IG	IN + IG	IN + IG	IN + IG
2 and 2u	IM	IM	IM	IM
3 and 3u	IM	IN + IG	IM	IN + IG

It is specified that the administration by the intramuscular route was carried out in the dorsolumbar muscles.

Antigens and adjuvants

Since there is a cross-reactivity between the GPLOs and *H. pylori*, it was chosen to use a preparation of inactivated *H. pylori* bacteria, as described in Example 1A, alone or in combination with recombinant urease prepared according to the method referenced in Example 1A.

~~The *E. coli* heat-labile toxin (LT) (Sigma) or the B subunit of the cholera toxin~~ (CTB) (Pasteur Mérieux sérums & vaceins) was used as mucosal adjuvant whereas DC-chol was used ~~as~~ parenteral adjuvant. DC-chol powder is simply rehydrated with an ~~antigen~~ preparation.

The doses used are as follows:

Route	Microorganisms	Urease	DC-chol	LT	CTB
IG	400 μg	2.5 mg	-	25 μg	-
IN	400 μg	400 μg	-	25 ng	25 μg
IM	400 μg	100 μg	400 μg	-	-

Biopsies, urease test, and bacteriological/histological study

A biopsy was performed on each of the monkeys before and after immunization (one month after the third booster). Using the biopsies, a urease test and a histological study were performed.

The urease activity is evaluated using the Jatrox kit (Procter & Gamble). The level of this activity is estimated as follows, in a decreasing manner: level 3, pink color appearing during the first 10 minutes; level 2, pink color appearing between 10 and 30 minutes after the addition of the reagents; level 1, pink color appearing between 30 minutes and 4 hours and level 0, weak or no color after 4 hours.

The histological studies were performed using biopsies fixed in formalin and the bacterial load was quantified as follows: absence of bacteria (0); a few bacteria of the *Helicobacter* type (0.5); fairly numerous bacteria (1); numerous bacteria (2); highly numerous bacteria (3). A difference of one level (for example from 1 to 2) corresponds to a number of bacteria 5 times greater.

Analysis of the response by the ELISA test

An ELISA test is carried out as described in Example 1A.

2B - Results

The table below relates to the bacterial load which, before and after immunization, is assessed using two tests: (i) by evaluating the urease activity and (ii) by carrying out a histological study. The results relating thereto are presented in columns 3 to 6. The last three columns indicate for each group (control, 1, 2, or 3) the

number of monkeys for which the bacterial load remains unchanged after immunization (→) according to the two tests; or appears lower (↘) or increased (↗) in at least one of the two tests, the other test indicating a stationary bacterial load. When the results of the two tests show a similar variation, the upwards or downwards arrow is double.

Monkeys	Group	Urease activity before immunization	Urease activity after immunization	Histology before immunization	Histology after immunization	Variation ↘	Variation ↗	Variation ↘
H 282	C	2-2	3-2	2	3-2			
J 005	C	2-2	2-1	2	1-0	1/4	1/4	2/4
J 852	C	0-0	2-0	0	1-1			(2/4↗↗)
J 476	C	0-0	2-0	0	1-1			
H 799	1	2-2	2-2	2	2-2			
J 845	1	2-2	3-2	2	2-1			
J 205	1	1-1	2-2	0	1			
J 328	1	2-2	1-2	3	3-2	1/8	5/8	2/8
J 197	1u	2-2	3-2	2	3			(1/8↗↗)
H 025	1u	2-2	2-2	1	1-1			
G 460	1u	2-2	3-2	3	2-3			
J 607	1u	2-2	2-2	2	2			
H 549	2	3-3	2-2	3	2-3			
H 622	2	3-3	1-1	2	2-3			
H 504	2	3-3	1-1	2	2-1			
H 798	2	1-1	0-1	1	1-1			
J 367	2u	2-2	2-1	3	2-3	6/8	1/8	1/8
G 486	2u	2-2	2-2	1	2-2			
J 522	2u	2-2	0-0	2	2-2			
G 722	2u	3-3	2-0	2	2-3			

H 820	3	3-3	2-2	3	2-2			
J 557	3	2-2	1-0	2	1-2*			
H 588	3	2-2	2-0	3	1-2			
J 153	3	3-3	3-3	2	3-3	5/8	0	3/8
H 480	3u	2-2	2-2	2	3-3	(3/8↘↘)		
J 344	3u	3-3	2-0	3	2-2			
H 710	3u	2-2	2-2	2	3-3			
J 262	3u	3-3	2-2	3	3-2			

Thus, this table reveals that in the group having been subjected to an immunization procedure by the strict mucosal route, the results are substantially identical to those obtained with the negative control group. On the other hand, in the groups having been subjected to an immunization procedure by the mixed mucosal and intramuscular route or by the strict intramuscular route, a marked reduction in the bacterial load is observed. This highlights the importance of the immunization conditions and in particular of the immunization rate; consequently, the use of an immunization procedure which employs the parenteral route targeted in the subdiaphragmatic region, is recommended in order to obtain a protective effect.

These results are to be placed in perspective with other results relating to the serum antibody levels which are presented in Figure 3. This figure shows that the immunization scheme by the strict mucosal route (1 and 1u) leads to results which are very similar to those of the negative control group. On the other hand, the immunization scheme by the mixed mucosal and intramuscular route (2 and 2u), and better still the immunization scheme by the strict intramuscular route (3 and 3u), makes it possible to induce antibody levels substantially greater than those of the control group.

Thus, a high serum response may be correlated with a protective effect, whereas *a contrario*, a low response is linked to the absence of a protective effect. The immunization conditions which make it possible to obtain the desired effect (high serum response and protective effect) include the use of the parenteral route targeted in the subdiaphragmatic region or that of a Th1 adjuvant.

Example 3: Treatment of an *H. pylori* infection in mice

We compared the efficacy of immunization *via* the subcutaneous (SC) route with that of the mucosal route, in order to treat an *H. pylori* infection in a mouse model.

OF1 mice were infected with 10^6 colony-forming units (cfu) of the *H. pylori*

strain ORV2001. After one month, verification that the infection was well-established was made by randomly sacrificing 10/100 mice and testing the urease activity on a quarter of the entire stomach. Since all of the results were positive, the mice were then immunized (10 per group) 3 times at 3 weekly intervals, either subcutaneously using 10 μg of recombinant urease supplemented with 15 μg of QS-21 (Aquila) or 400 μg of adjuvant Bay R1005 (Bayer), or orally using 40 μg of urease mixed with 1 μg of LT. For each of the two adjuvants administered parenterally, the immunization was carried out either in the neck, in order to reach the lymphatic ganglions of the upper region of the body, or in the lumbar region, in order to reach the abdominal lymphatic ganglions. Ten mice were left uninfected and unimmunized (negative control), whereas the mice of the positive control received a saline solution, QS-21, or Bay adjuvant subcutaneously (lumbar region).

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~~One month after the~~ third immunization, all of the mice ~~were sacrificed and~~ the stomachs removed to evaluate the extent of the colonization by measuring the urease activity (10/10 mice were analyzed in each group), as well as by carrying out quantitative culturing (5/10 were analyzed). Figures 6A (test relating to urease) and 6B (culturing) show that in the mice immunized with urease supplemented with QS-21, subcutaneously in the lumbar region, the infection had virtually disappeared (4/5 mice were negative in quantitative culturing). The mice immunized with urease subcutaneously in the neck, in the presence of QS-21, and the mice that received urease plus LT orally exhibited a 10- to 100-fold decrease in the infection when compared with the unimmunized mice. The Bay adjuvant induced an identical decrease, which was more pronounced in the mice ~~immunized in the~~ lumbar region.

Histopathology carried out on these same mice did not reveal any gastritis that was more extensive than in the controls.

As we observed in our previous prophylactic study (Example 1), the protected mice had a high level of the two isotypes IgG1 and IgG2 in the serum, which is representative of a Th2/Th1 equilibrated response. Furthermore, the mice immunized

subcutaneously in the lumbar region had the highest levels of IgA in the serum, which demonstrates a mucosal response.

These results show that targeted, systemic immunization is capable of curing an acquired *H. pylori* infection in mice, and that the use of adjuvants that induce an equilibrated mucosal response of Th1/Th2 type is desirable in order to achieve this aim.

Example 4: Mucosal prime/parenteral boost strategy for urease immunization that elicits protection in mice against infection with *H. pylori*

Swiss Webster mice were immunized with a mucosal prime/parenteral boost strategy. A single oral dose of 25 μg recombinant *H. pylori* urease (urease) and 5 μg *Escherichia coli* heat labile enterotoxin (LT) was administered as a prime. Three weeks later, mice were boosted by the parenteral route with 2 doses, 3 weeks apart, with 100 μg urease + 100 μg alum. Mice immunized by this prime/boost strategy exhibited a 2,000-fold reduction in the median *H. pylori* colony forming units (CFUs) compared to unimmunized controls (Figure 7). Immunization by this strategy was more efficacious than 3 doses of mucosal vaccine when a 1,000-fold reduction in colonization was achieved. A single priming dose of orally administered urease + LT only produced a 10-fold reduction in median *H. pylori* CFUs, boosting with urease administered parenterally without an adjuvant resulted in only a 2-fold reduction in medium CFUs (Figure 7).

This immunization strategy was used to immunize rhesus monkeys against challenge with *H. pylori*.

Example 5: Protection of rhesus monkeys from *H. pylori* infection by urease immunization using a mucosal prime/parenteral boost strategy

Rhesus monkeys, seronegative for *H. pylori*, were treated with quadruple therapy and confirmed to be free of *H. pylori* infection by culture and histologic

examination of gastric biopsies. Nineteen monkeys were randomized into 4 groups: 4 monkeys were sham vaccinated, 5 received recombinant *H. pylori* urease (urease) vaccine given by the intramuscular route with alum (aluminum hydroxide, Rehydragel) as an adjuvant, 5 received a mucosal priming dose of urease vaccine given by the oral route with *Escherichia coli* heat labile enterotoxin (LT) as an adjuvant, followed by two parenteral boosts of urease vaccine given by the intramuscular route with alum, and 5 monkeys received urease vaccine given by the intramuscular route with Bay adjuvant. Monkeys were immunized every three weeks for the first three immunizations, and a fourth dose was administered at 20 weeks.

One week after the last immunization, the monkeys were challenged with *H. pylori*. The monkeys were sacrificed 10 weeks after challenge. At necropsy, 10 gastric sites (1 cardiac, 2 corporal, 3 corporal-antral junction, 3 antral, and 1 pyloric) were sampled for bacterial culture by taking 5 mm diameter punch biopsies. Additional tissues from the 5 regions of the stomach were harvested for histopathology.

Although all of the monkeys were infected with *H. pylori* as a result of the experimental challenge, monkeys immunized with urease + LT as a mucosal prime followed by 3 parenteral booster doses of urease + alum showed statistically significant reduction ($p=0.05$, Wilcoxon rank sums test) in colonization when compared to the control, sham immunized monkeys (Figure 8). A greater than 20-fold reduction in median bacterial colony forming units per bunch biopsy (5.8×10^2 CFU, ranging from 1×10^2 to 6.7×10^2 CFU) was found in monkeys that received the vaccine as a mucosal prime/parenteral boost regimen, compared to a median CFU of 1.3×10^4 (range 1.5×10^3 to 1.8×10^5 CFU) for the group of sham immunized monkeys.

The group of monkeys receiving urease vaccine in a mucosal prime/parenteral boost regimen had similar gastritis (Figure 9) and epithelial changes (Figure 10) after challenge with *H. pylori*. There was no evidence that vaccination enhanced either

gastritis or epithelial alterations.

~~In contrast to the monkeys~~ receiving the mucosal prime/parenteral boost regimen, monkeys immunized by the parenteral route with urease + Bay showed no difference in *H. pylori* colonization compared with the sham-immunized controls (p = 1.00), while monkeys treated with urease + alum showed a partial effect (p=0.33) (Figure 8). Culture data was unavailable for one of the monkeys in the group receiving urease + Bay, due to heavy contamination of gastric samples with other bacteria.

These results show that an immunization scheme utilizing a mucosal prime of urease + LT followed by parenteral boosting with urease + alum is efficacious in preventing *H. pylori* infection in non-human primates. This scheme was chosen with the rationale that the immune system must be primed to respond to a mucosal infection, *i.e.*, by mucosal immunization with an appropriate adjuvant. However, once the immune response is 'set' properly, a more conventional adjuvant, such as alum, can be used as a parenteral immunization to boost the response. This kind of scheme is not only more effective than a mucosal only regimen, but it utilizes less antigen because the parenteral antigen dose (100 μg urease) can be much less than a mucosal dose (4 mg).

Other embodiments are within the following claims.